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POLLEN AND POLLEN ENZYMES

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I. THE THEORETICAL AND PRACTICAL ASPECTS OF THE OCCURRENCE OF POLLEN ENZYMES

Reasons for Undertaking the Investigation

A review of the literature shows very few complete or satisfactory reports of experiments in regard to either the general chemistry or the enzymes of pollen. Our knowledge of the subject seems to be very fragmentary. It is conspicuous by its omission from the textbooks of botany. Aside from the few references given later, up to the present time no mention of any important work has been found.

Although it is generally assumed, and is stated in our textbooks, that the pollen tube digests its way through the tissues of the pistil and the ovule, yet there seems to be no experimental evidence as to the exact nature of this enzyme action. Besides this, pollen enzymes must be very important in rendering the food stored in the grain available when the pollen germinates, in nourishing the tube during its passage through the style, and in stimulating the development of the embryo and the maturing of the ovary.

Moreover, pollen anaphylaxis is now regarded as the cause of so-called hay fever and other forms of pollen poisoning. Pollen enzymes may be concerned in these reactions, and the proteolytic enzymes may affect the stability of the pollen-protein solutions used in pollen vaccination.

In view, therefore, of the apparent meagerness of our knowledge of pollen enzymes and of the possible practical value of any contribution to this subject, it has seemed worth while to study the matter and to present the results.

The Literature of Pollen Enzymes

Few original, systematic experiments have been reported. Erlenmeyer (1874) found amylase, or diastase, in pine pollen. Van Tieghem (1869) reported invertase, or invertin, in the pollen of hyacinth, narcissus, wallflower, and violet. Czapek (1905, p. 393) quotes Strasburger's statement

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that the pollen tubes of *Agrostemma Githago* bore through the membrane of the stigma papillae as evidence for a cytase in pollen. Czapek also refers to the investigations of Rittinghaus (1886, pp. 105–122) as confirming the opinion of Strasburger. The observations of Rittinghaus may, however, be interpreted quite differently, and point quite as definitely to the presence of a pectinase, as of a cytase. Rittinghaus examined numerous flowers, including Ipomoea, Convolvulus, Alisma, Agrostemma, Lychnis, Phlox, and Silene. He writes (p. 111):

> Die Verschmelzung zwischen der Cuticula der Papille und der Cellulosemembran des Schlauches ist ganz deutlich zu erkennen, und es leuchtet ein, dass die Lücke in der Cuticula ihre Entstehung nur einer unmittelbaren Einwirkung der Pollenschlauchspitze verdankt. Das lösende Agens ist somit nur im Plasma des Pollenschlauches zu suchen. Über die Natur desselben ist einstweilen leider nichts zu eruiren, zumal das einzige uns bekannte Cuticula-lösende Reagens kochende Kalilauge ist. Vielleicht wird man später die Erscheinung durch die Gegenwart eines besonderen Enzymes aufklären können.

J. R. Green (1891) noted amylase in pollen tubes. Green's later researches in 1894 are by far the most careful and complete experiments on pollen enzymes which have so far been reported. They will be briefly reviewed on a later page. Strasburger (1886) mentions diastase and invertin as present in pollen grains prior to germination. Sandsten (1909) reports invertase and diastase. Later, Kammann (1904) found protease, diastase, catalase, and lipase in rye pollen but does not give details of his experiments.

In the investigations of Green (1894, pp. 385–409) the pollen was powdered with glass and the powder suspended either in glycerine, or in a 5 percent solution of NaCl, to which 2 percent of potassium cyanide was added as an antiseptic. In other cases chloroform (a few drops) or oil of cinnamon was used as an antiseptic. The 5 percent NaCl solution proved preferable to glycerine. Diastase was found in the pollen of Gladiolus, Anemone, Antirrhinum, Tropaeolum, Pelargonium, Crocus, Brownea, Helleborus, Alnus, Tulipa, and Clivia; also in that of Zamia after germination begins. Experiments failed to show any sufficient evidence for diastase in the resting pollen grain of Zamia, and starch makes its appearance in these pollen grains only on germination. Diastase was absent from the pollen of Lupinus, Lathyrus, Eucharis, Richardia, and Narcissus. The diastase, according to Green, dissolves the starch without corroding the grains. The pollens tested for invertase were those of *Eucharis grandiflora*, *Narcissus papyraceus albus*, *N. Pseudo-Narcissus*, Helleborus, Richardia, *Lilium pardalinum*, and *Zamia Skinneri*. It was found in these, but was absent from the pollen of Alnus and of Clivia. He reports that

> A few experiments were made with a view to determining the existence of a cytolyst and a proteolyst, but in no case could either be found.

In the case of *Eucharis grandiflora*, tested for invertase, Green says that

> Only the contents of three or four anthers were used, yet a workable quantity of invertase was extracted.

In summarizing he says:

> The enzymes present in the resting pollen grains are, therefore, chiefly diastase and invertase, but their distribution is irregular, some containing one, some the other, and some both. At the onset of germination usually the amount of both diastase and invertase is considerably increased. . . . When the grain has lost the power of germinating the quantity of diastase is materially decreased.

The conclusions, as will be noted later, are not entirely in accordance with the results of the present experiments.

The Significance of Pollen to the Living Plant, and the Probable Rôle of the Pollen Enzymes

A medium-sized Indian-corn plant produces about 50,000,000 pollen grains. Cat tails (Typha), which produce about 60,000 flowers to the average spike, shed enormous quantities of pollen. A near relative, the elephant grass (*Typha elephantina*) of East India and New Zealand, yields enough for the natives to use as a flour in bread- and cake-making. The dense cloud of pollen from a pine tree has been photographed, and many a camper has noticed the yellow powder staining the canvas of his tent when dampness has moistened the grains. Liefmann (1904, p. 163) found 2,500,000 grains of grass pollen in one square meter. Yet so tiny and light are these pollen grains that a small amount represents millions of grains. Ulrich (1914) estimated 172,800,000 grains in one gram of ragweed pollen, and Kammann (1912) estimated 20,000,000 in one gram of timothy pollen. Pollen grains are nearly omnipresent during the flowering season. One would suppose from these figures that it is an easy matter to collect large quantities of pollen, but it is really not easy. The winged grains of pine pollen are blown away by the slightest breeze. Ragweed pollen cannot be collected easily after nine o'clock in the morning. The grain of pollen is surrounded by an oily envelope containing air. When this air is heated by the sun it causes the floating away of the pollen, or the so-called "smoking" of the ragweed. It is not easy to get enough for an experiment. The fact that during three fourths of the year we have pollen grains always with us makes it evident that if they have active enzyme action their importance cannot be lightly overlooked.

Pollen grains present many types of configuration. The commonest forms are oval or spherical, but an extreme variation is seen in the extraordinary filamentous pollen grains of eel grass (Zostera) and of another water plant, Halophila. Although the grains differ greatly in shape and in surface markings or finish, in internal structure they are very uniform. They usually consist in the Angiosperms of two cells. One cell is purely vegetative and gives rise to the pollen tube; the other is the generative cell.

Pollen grains vary considerably in size. A very extensive list of both measurements and descriptions of the pollen grains of many species and families is given by Hansgirg (1897, pp. 17–76).

The pollen grains are very resistant to excessive heat, cold, or dryness, and certain kinds retain their viability for many years. The pollen of the date palm tested by Popenoe at the Mecca experiment station was kept seven years and still retained its power of germination. Goodale (1916) found that dry pollen could retain its active poisonous properties for twenty-five to thirty years. It is evident that pollen is an interesting physiological unit, and our knowledge of its composition should be more complete.

Since one cell of the pollen grain is vegetative and gives rise to the pollen tube, food must be stored in the grain and at the time of germination rendered available. We should expect therefore to find enzymes suitable for the digestion of the materials stored in the grain, and perhaps capable of also digesting the inner pectin membrane (Mangin, 1893, p. 655) which envelops the grain. It is one aim of the experiments reported to determine whether such a correlation exists.

The distance that the pollen tubes have to traverse varies greatly. Where a style is absent and the stigmatic surface is just above the ovary, as in Vitis and Actaea, the tube has only a little way to penetrate. In flowers with long tubular corollas and slender filamentous styles, such as Crocus, Oenothera, and *Zea Mays*, the tubes attain a relatively great length. The time required for them to reach the ovule also varies greatly. In some flowers the tube reaches its full development in a few hours, while in the pine, following pollination in the spring, the grains put forth short tubes which do not complete their growth for a year (Kerner, 1895, **2**: 420). In certain oaks thirteen months elapse between pollination and fertilization. In regard to the Taxaceae, Coulter (1910, p. 268) writes:

> The tube may advance directly toward the archegonia or it may pursue a devious route, in some cases not reaching the archegonia until during the second season.

Other instances are cited by Coulter and Chamberlain (1903, p. 147). Why this long delay? An interesting physiological and chemical problem is waiting to be solved. The 13-inch pollen tube of *Colchicum autumnale* needs only twelve hours to reach its goal, and the 9-inch tube of *Cereus grandiflorus* completes its growth in a few hours (Schleiden, 1849, p. 407). In *Iris versicolor* the male nuclei were observed in the embryo sac 79 hours after fertilization and the tubes were 14 mm. long (Sawyer, 1917, p. 163). Surely an intruding, growing tissue of such size and duration must during its period of development, profoundly affect the cells with which it comes in contact, or which are adjacent to it, in its passage through the style. It has long been customary to liken the pollen tubes to the haustoria of parasitic fungi, for they closely resemble the latter in many respects. In Pinus, according to Mottier (1904), the tube serves both as a conducting passage for the male gamete and as an absorber of nutriment. The haustorial habit seems to be the more primitive condition, and we have survivals of it in certain Angiosperms, as in *Iris versicolor* (Sawyer, 1917), hazel, oak, elm, hickory, and certain mallows (Kerner, 1895), where the tube branches

frequently and serves apparently as both haustorium and directing channel. (See also Coulter and Chamberlain, 1903, p. 148.) The nature of the tube has been dwelt upon here at such length in order to emphasize the fact that we ought to know more fully how these tubular filaments make their way through the tissues of the style and ovary. We assume that they digest their way. One author of a recent textbook even states positively:

> Very soon after pollination, the tube cell begins to develop a pollen tube, *which secretes an enzyme that dissolves the cell walls* and contents of the nucellar tissue, thus facilitating the passage of the delicate tube.

Is this true? Can we prove the existence of a cytase which digests the cell wall? Is one enzyme sufficient to account for the varied needs of the pollen tube in the course of its life history?

There are several conditions which the pollen tubes may encounter before they reach the embryo sac. These are as follows:

(1) *An open stylar canal.* In such cases the germinating tubes may force apart the cells of the stigma and soon enter the open space of the style without having to penetrate any cells, at least not until they reach the ovule. The middle lamella is usually composed of pectin compounds (Frémy, Mangin, Allen, and others). A pectin-digesting enzyme might therefore be required to dissolve the middle lamellae of the stigmatic cells, but afterwards the tube has a clear course. Examples of this sort are seen in violet, mignonette, lily, rhododendron, Hypericum, Cistus, *Atropa belladonna*, and iris. According to Kirkwood (1906), in the Cucurbitaceae

> The tubes pass chiefly over the surface of the conducting tissue lining the stylar canal and covering the placenta lobes, and this is rich in starch.

The suggestion is made that the tube is directed in its course by nutrient substances secreted by the conducting tissue. This would imply the presence of a diastase to digest the starch. Even if there is actually no tissue to be digested, it seems reasonable to suppose that the tubes may derive nourishment from the cells lining the stylar canal. Negative aërotropism, positive hydrotropism, and positive chemotropism, which have been frequently demonstrated in pollen tubes, direct their course so that they penetrate the stigma. These same responses tend in many cases to keep the tubes closely appressed to the cells lining the canal. Considering the length of time it often takes a tube to reach the ovule and its considerable growth, enzymes along with other factors in nutrition must play an important part. Frequently, as in Anagallis, the channel is only a narrow space almost completely filled with a mucilaginous substance, supposed to be secreted by the cells lining the canal. It may be pointed out here that the mucilages are closely related to the pectins. If this material is utilized by the tubes during their passage through it, we should expect a suitable enzyme to be present.

(2) *A mass of loose, conducting tissue in the style.* The cells in the interior

of the style frequently are loosely connected, elongated, and sometimes mucilaginous. The pollen tubes, according to most histological reports, penetrate the middle lamellae of these cells. This is the condition most frequently met with. The pollen tubes follow the middle lamellae of the cells throughout their course. The lamellae are, as has already been stated, composed either of pectin or of closely related mucilaginous substances. Here again the necessity for a pectin-digesting enzyme is evident. It has been sought for in the experiments reported later. Since this condition is the most common, many examples could be cited. It may be well seen in members of the grass family and in Salvia (Bower, 1919, p. 269). Histological evidence seems to indicate that the cells of the style often remain intact. Shreve (1906, p. 115) says in regard to the pitcher plant (*Sarracenia purpurea*):

> The pollen tubes grow between the cells of the stigmatic surface and their entire passage is between the cells of the conducting tissue and never through them.

Gow (1907, p. 136), describing the fertilization of skunk cabbage (*Spathyema foetida*), writes:

> The central portion of the style consists of a loose mass of thin-walled cells through which the pollen tube readily forces its way to the upper end of the ovary.

Miller's account of the growth of the pollen tube of corn through the silk or style is interesting (1919, p. 264):

> Each silk has two fibro-vascular bundles. These bundles are surrounded by sheath cells which are characterized by their dense contents and large flattened nuclei. It is *between* these cells that the pollen tube travels down the silk. Arriving at the base of the silk the pollen tube works its way *between* the sheath-like cells that extend from the fibro-vascular bundles of the silk to the cavity of the ovary. The tube enters the ovary and twists and coils in its passage along the ovule coat until it reaches the micropyle. The pollen tube then pushes *between* the cells of the ovule until it reaches the embryo sac.

Again, in another part of his account, he says:

> The end of the pollen tube is greatly enlarged as it pushes its way between the sheath cells of the bundle. In its passage down the silk the *tube causes but little disturbance in the position of the cells, so that after the tube disappears the cells quickly return to their normal form and position.* [The emphasis here is my own.] The pollen tube so far as I have observed does not extend the full length of the silk at any time. It is difficult to locate it a short distance back of its growing region. It appears that the older portions of the tube are absorbed by the surrounding cells, while the growing part of the tube is apparently nourished by the dense sheath cells.

Land (1907, p. 276), in explaining the fertilization of *Ephedra trifurca*, notes that the pollen tubes force their way between the neck cells of the archegonium, rarely destroying them in their passage. Only in two instances were the lower neck cells destroyed.

(3) *Cell walls penetrated by pollen tubes.* According to most investigators this condition occurs only rarely. Perhaps it will be found more frequent if more observations are made. The classic illustration is corn cockle,

Agrostemma. Strasburger's illustration of the tubes actually penetrating and half filling the papillar cells of the stigma has been frequently copied. Mallow pollen tubes do the same. Recently Knight (1918, entry 964) has reported that in the apple there is no stylar canal. "Pollen tubes make their way through the tissue. There is a decomposition of the cells along this path with the extrusion of mucilage." This is interesting to compare with the opinion of Grieg Smith that mucilages are decomposition products of cellulose, and with Wiesner's statement that all gums are produced by a diastatic ferment acting on cellulose. The writer regrets that it has been impossible to secure corn cockle and mallow pollen so as to determine whether their enzyme action is different from that of other pollens. Apple pollen has shown some differences. In histological studies of fertilization little attention seems to have been paid to the question of how much the pollen tube disorganizes the neighboring cells. It seems that it would be worth while to examine material again with this thought in mind. Many of the drawings of the passage of the pollen tubes appear very diagrammatic. In this connection it is interesting to note Kerner's observation (1895, p. 392) that the pollen tubes of *Lamium amplexicaule*

> Perforate the walls of the anther and grow in the direction of the stigma until they reach it.

Pollen Grains as Carriers of Bacteria and Molds

Nine varieties of pollen were tested to see if any contained a rennin-like enzyme, such as is found in the juices of a number of plants. Thymol had been added to the unheated and autoclaved pollen extracts, but the milk had not been sterilized. It was observed that both the unheated ragweed pollen and the autoclaved dock pollen control had strongly coagulated the milk over night at room temperature. Repetition of the test with highest grade milk (Fairlea Farm) showed that unheated corn, Easter lily, and dock pollens caused clotting, as did even the autoclaved dock pollen. The strong "youghourt" or fermented milk odor, and the behavior of dock pollen made the reaction seem more like bacterial than like enzymatic action. Apparently the single period of heating in the autoclave had not destroyed all bacteria on dock pollen. Accordingly a number of tests were made employing the usual bacteriological methods. These tests showed that pollen grains harbor a varied flora of both bacteria and molds. It had been taken for granted that excess of toluol or of thymol was sufficient to inhibit bacteria and molds. Do the results of these tests with milk mean that in other instances it is the enzymes of bacteria and molds rather than those of pollen grains which cause the change? The writer believes that this is not true for the following reasons:

a. The results were constant with the same pollen regardless of its source. Corn, pine, maple, and goldenrod pollen were collected both in New Haven, and, owing to the difference in seasons, a few weeks later on

the hills of Vermont, six miles from a town. When this possible source of error was suspected, ragweed pollen was purposely obtained from Michigan, from two parts of New York state, and from Connecticut. It does not seem probable that the bacteria and molds carried by pollen can be so constant as to cause similar enzyme action in each instance.

b. The reactions are too rapid to be due to bacteria. With the inhibiting action of antiseptics the time required for bacteria to develop in sufficient numbers to produce similar changes would be much longer. All the enzyme reactions recorded have occurred within 24 hours, and several have been almost instantaneous.

c. Slices of wood in water over night are not in any degree sterile, yet bacteria which have free access do not destroy the middle lamellae, but pollen grains do. Pollen grains taken from unopened anthers and put into sterile Petri dishes are not likely to have peculiar bacteria, absent from the immediate environment. Besides, examination of the pollen contamination showed only a few omnipresent common forms of bacteria.

d. Pollen solutions filtered through a Berkefeld filter gave the enzyme action of diastase on starch, and blood fibrin digestion.

e. It is probable that the ground pollen added something to the milk which stimulated the growth of bacteria already in the milk, and that it was these which caused coagulation rather than the bacteria introduced by the pollen. The reason for this belief is that in all the plates poured from milk to which pollen had been added *Bacillus fluorescens liquefaciens* was the dominant type. The plates after standing a few days were a bright apple-green from the fluorescent growth.

On other plates poured later from the pollen extracts only, not once did this form appear. In the latter it was often not until the third or fourth day that colonies of molds occurred. Doubtless there are resistant forms of spores on the pollen which endure the heat of the autoclave and develop under favorable conditions on the agar plates, but these can hardly account for digestions which occur during twenty-four hours.

The Chemistry of Pollen

While many kinds of pollen have been examined for certain special constituents such as starch, nitrogen, phosphoric acid, etc., only eight kinds of pollen, as far as I have been able to ascertain, have been analyzed with any degree of completeness. Czapek (1905) discusses topically the occurrence and distribution of the principal constituents of plants; if a substance has been reported present in pollen he mentions the fact. These scattered references afford a valuable index to the original literature of the earlier analyses.

According to Heyl (1919*a*, p. 672) the walls of the pollen grain constitute 65 percent of the structure. Biourge (1892, p. 75) distinguishes four substances in the wall or envelope of pollen grains: cutin, cellulose, pectic

substances, and callose. Sometimes one, or more than one, of the four materials are present in the same grain. These substances are indicated by characteristic solubility tests and by color reactions. He examined the pollen of 19 species of monocotyledons and 26 species of dicotyledons. His plates give over a hundred illustrations of the pollen grain coats and their sculpturing, showing details brought out by staining methods and chemical treatment. Mangin (1888, p. 144) states that the membrane is formed of pectin.

Water makes up a large but variable part of the grain. Thus Koessler (1918, p. 420) found 10.5 percent of moisture in ragweed pollen, while Heyl (1917, p. 1470) reports 5.2 percent for the same kind of pollen. Braconnot (1829, p. 104) found 47 percent of water in cat-tail pollen. Lidforss (1899, p. 292) examined a number of species and found the average moisture content to be about 10 percent.

The colors of pollen differ greatly. It is deep yellow in Easter lily, dark red in tiger lily, salmon in cypress, and white in petunia. Even in the same flower the color may vary, as is noted by Plimmer (1912, p. 51) in *Lythrum salicaria*, which has yellow pollen in the short stamens and bluish green pollen in the long stamens. Heyl (1919 *b*, p. 1285) states that the yellow pigment of ragweed is entirely glucosidic and about 0.6 percent of the pollen. He finds a quercitin glucoside which on melting yields a cherry-red oil; and a glucoside isorhamnetin which has beautiful characteristic crystals in the form of hexagonal prisms. So far, no other analysis of the pigments of pollen has been located in the literature.

Starch has been found present in some kinds of pollen and absent in others. Molisch tested 110 varieties and found starch abundant in 45, only a trace in 9 varieties, and absent from 46. That is, about half the kinds tested contained starch. Lidforss (1899, pp. 294–298) examined 150 wind-pollinated flowers of 72 genera and 29 families of native or naturalized Scandinavian plants, and found the pollen of all rich in starch. On the other hand, he tested the pollens of a few wind-pollinated tropical plants and found them starch-free. He also calls attention to the fact that Nägeli found the pollens of *Alnus glutinosa* and *Plantago lanceolata*, collected in Germany, starch-free, while pollens of the same species collected by himself in a more northerly region contained starch. Similarly, Nägeli found the pollen of juniper on Swedish mountains to be rich in starch, while Molisch found little in that of the Austrian juniper. Further, Molisch states that the pollen of *Antirrhinum tortuosum* is completely starch-free in summer, but in November he finds grains of three sorts, those which are normal but starch-free, little empty grains, and normal starch-containing grains. Tischler (1909, pp. 219–242), however, does not find this correlation between climate, or temperature, and the starch content of pollen. He examined a large number of tropical plants at Buitenzorg and reports that the plants growing under relatively unfavorable conditions of assimilation, for example

on mountains 3,000 m. high and in the desert, showed no higher percentage of pollen with starch than the plants growing under the favorable climatic conditions of the tropical rain forest. He does, however, observe that there is frequently a difference in starch content between mature and immature grains.

Lidforss (1899, p. 306) reports the analysis of sixteen varieties of pollen for nitrogen and P_2O_5. Of these, 11 were from anemophilous, and 5 from entomophilous flowers. He found the average nitrogen content of the wind-carried pollen to be 4.63 percent, while that of the insect-carried pollen was 7.49 percent. The P_2O_5 showed a similar difference; the average for the former pollen being 1.76 percent, and for the latter 3.03 percent. Whether or not this represents a real correlation must be established by further observations.

The relative amounts of protein, fat, sugar, ash, etc., can best be seen by comparison of tables 1–7. It is interesting to note, from Stift's analyses of the pollen of three varieties of *Beta vulgaris*, that the different constituents may vary considerably in the pollen of one species (Stift, 1896, p. 43; 1901, pp. 105–106).

TABLE 1. *Comparison of Pollen Analyses (figures indicate percentages)*

Kind of Pollen	Authority	Protein	Fat	Ash	Carbohydrates
Date palm..	Vauquelin, 1802			$Ca_3(PO_4)_2$ $Mg_3(PO_4)_2$	
Cat tail.....	Braconnot, 1829		3.60		Starch Sugar
Cypress....	Church, 1875	8.67	1.87	3.70	85.76
Hazel.....	Planta, 1885	30.06	4.20	3.81	5.26 14.7 Saccharose
Pine.....	Planta, 1885	16.56	10.63	3.30	7.06 11.24
Pine.....	Kressling, 1891	15.87	10.00	5.50	7.40 12.075 Pentosans
Beet.....	Stift, 1896,	16.90	3.52	9.18	0.89 12.26
	1901	16.68	5.47	7.13	0.89 7.27
Rye.....	Kammann, 1912	40.00	3.00	3.40	25 Dextrin Sugars
Ragweed ...	Heyl, 1917	24.40	10.80	5.39	2.10 2.10 Pentosans 7.26
Ragweed ...	Koessler, 1918	8.25(?)	10.30	10.60	6.89

Stoklasa (1896, p. 631) analyzed the pollen as well as various other organs of apple, horse chestnut, and beet, and concludes:

> Das lecithinreichste Organ der ganzen Pflanze aber ist entschieden das Pollenkorn.

He found in apple pollen 5.86, in that of horse chestnut 5.16, and in that of beet 6.04 percent of lecithin. Heyl (1919*a*, p. 672) discusses the chemical "building stones" from which the substance of pollen sperm nuclei may be

built, if there is a parallelism with the chemical composition of animal sperms.

TABLE 2. *Analysis of Pine Pollen, Przybytek and Famintzin, 1885 (figures indicate percentages)*

Water	6.79
Ash	
Calcium oxid	35.23
Sodium oxid	3.62
Magnesia	7.00
Calcium	0.88
Iron and aluminum oxid	5.30
Phosphoric acid (anhydrous)	29.86
Sulphuric acid (anhydrous)	14.83
Chlorine	0.99
Manganese	a trace

TABLE 3. *Stift's Analyses of Pollen from a Cattle-fodder Beet and from two Varieties of Sugar Beet (figures indicate percentages)*

	Fodder Beet, 1895	Sugar Beet, 1895	Sugar Beet, 1900
Protein	15.25	16.90	16.68
Nitrogenous substances not protein	2.50	2.77	5.82
Fat (ether extract)	3.18	3.52	5.47
Starch and dextrin	0.80	0.89	0.89
Pentosan	11.06	12.26	7.27
Other nitrogen-free extractives	23.70	26.27	28.86
Crude fiber	25.45	28.21	27.95
Ash	8.28	9.18	7.13
Water	9.78		

TABLE 4. *Heyl's Analysis of Ragweed Pollen (1917)*

Alcohol-soluble (42.9 percent) contains (in percentages):

Moisture	5.28
Starch (diastase)	0.00
Crude fiber	12.20
Pentosans	7.26
Protein	24.40
Nitrogen in alcoholic extract	1.08
Ash	5.39
Dextrin	2.10
Fat	10.80
Lecithin	0.75
Ether-soluble, but not ligroin-soluble	1.75
Sucrose	0.40
Glucose	1.60
Resin	17.40
A nitrogenous base	trace

From the above review and from the analyses given in tables 1–7 it is clear that our knowledge of the chemistry of the pollen of the very numerous species of flowering plants is very limited. It is a discouraging problem

because of the difficulty of getting large quantities of material, as Heyl points out when he estimates that it takes 610 million grains of ragweed pollen to make a gram.

TABLE 5. *Kammann's (1912) Analysis of Rye Pollen (figures indicate percentages)*

Inorganic substances		13.58
Water	10.18	
Ash	3.4	
Organic substances		86.42
Alcohol-ether-soluble	3.	
Carbohydrate	25.	
Non-protein nitrogen	18.	
Protein	40.	

TABLE 6. *Koessler's (1918) Analysis of Ragweed Pollen (figures indicate percentages)*

Inorganic substances		21.1
Moisture	10.5	
Ash	10.6	
Organic substances		78.9
Total reducing sugars after hydrolysis	6.89	
Ether-soluble lipoids	10.3	
Fatty acids after hydrolysis	4.75	
Phytosterol	0.34	
Insoluble in ether but soluble in 95 percent alcohol	12.5	
Extractives, etc., soluble in alcohol (resins) and water	11.5	
Insoluble residue (crude fiber, proteins, etc.)	37.71	

TABLE 7. *Purin Bases and Amino Acids in Pollen*

Kind of Pollen	Authority	Purin Bases	Percentage
Pine	Planta, 1885	Hypoxanthine Guanine	0.04
Hazel	Planta, 1885	Hypoxanthine Guanine	0.15
Pine	Kressling, 1891	Xanthine Guanine Hypoxanthine	0.015 0.021 0.085
		Amino Acids	
Ragweed	Heyl, 1917	Histidine Arginine Lysine Agmantine	not given " " "
Ragweed	Koessler, 1918	Arginine Histidine Cystine Lysine	2.13 2.41 0.57 0.97

Other Physiological Aspects of Pollen in which Enzymes may Play a Part

In certain flowers there are two kinds of pollen grains, some of which produce tubes and others which do not. Müller (1883, p. 242) first distinguished these as "*Befruchtungs*"- and "*Beköstigungs*"-pollens, the former being the fertile, and the latter the sterile pollen which Müller

thought served as the food of the pollinating insects. Tischler (1910, pp. 219–242) has studied this subject and has made the interesting discovery that in certain pollens, at least, the sterile grains may be stimulated to produce tubes by the addition to the culture medium of a trace of saliva or of diastase. The lack of a specific enzyme in these pollens seems thus to be the cause of sterility. It is quite possible that in other pollens the lack of pectinase, cytase, invertase, or of other enzymes may be equally important in inhibiting the growth of the tube. In some cases the deficiency may be made good by an enzyme secreted by the stigma. The whole question has a great deal of significance in problems of plant breeding.

Pollen enzymes may be concerned in the production of the characteristic odors of pollen which are probably factors in insect attraction. The emanations from moist pollen indicate the presence of fermentation products.

It also seems reasonable to suppose, as Erlenmeyer (1874, p. 206) has suggested, that pollen enzymes are co-workers with the enzymes from the body of the bee used in producing bee-bread.

Gardeners commonly believe that contact with pollen is frequently the cause of the discoloration and decomposition of the petals which is often a sequence of pollination.

II. Experiments in Regard to Pollen Enzymes

Plan of the Experiments

An effort has been made to collect a large variety of pollens, representing different families of plants, and including some of the so-called "hay-fever pollens." These pollens have been tested for twelve different enzymes. On account of the difficulties in collecting all the pollens at the start, the experiments have been made in two series. For the first the available pollens were those of (1) Easter lily, (2) *Lilium rubrum*, (3) red maple, (4) Norway maple, (5) Siberian crab-apple, (6) Austrian pine, (7) Scotch pine, (8) magnolia, and (9) dandelion. In the second series of experiments, in addition to some of the first nine pollens, those of the following plants were used: (10) corn, (11) daisy, (12) dock, (13) elm, (14) goldenrod, (15) ragweed, (16) rye, (17) tiger lily, (18) timothy. Not every one of the eighteen pollens has been used in every test, but an effort has been made to use as many as possible.

Methods of Collecting Pollen. Kinds of Pollen Used

The work was begun in February. At this time Easter lily pollen was available in the largest quantity. Since it is customary to remove the anthers as the flower opens, to prevent the pollen from staining the petals, it was easy to find an obliging florist who would place these anthers in a clean paper box. In this way surprisingly large quantities of pollen were secured. Care had to be taken to prevent molding. A paper box was

found to be better for collection than glass jars, as the anthers dried more readily. It was also necessary to keep the anthers spread out, and to place them in a sulphuric-acid desiccator as soon as possible after collection.

When the anthers are dry, or partially dry, the large, sticky yellow pollen grains easily fall out. They can then be accumulated quickly by placing the anthers on one half of the bottom of a petri dish, moistening the other half with the finger tip, and then when the dish is covered and shaken in a horizontal plane the pollen adheres and heaps up on the moistened surface.

When it was necessary to remove adhering masses of pollen from a dish a glass brush was found better than a camel's hair brush, and for this purpose the glass brush from a Beegee ink eraser was excellent.

The easiest way of collecting the tiny pollen from many small flowers is by drying the blossoms on large sheets of paper and shaking them through a fine sieve. The anthers usually sift out and the pollen can be separated from the anthers by sifting again through fine silk bolting cloth. (Mimeograph typewriter diaphragm silk is convenient.) The microscope showed, in the case of red maple, that invisible hairs from the flower also sifted through, but the pollen from other plants appeared quite free from foreign particles.

Wodehouse (1916, p. 430) has suggested an excellent way of collecting large quantities of ragweed pollen.

> The flower heads just coming into bloom are crushed in a mortar with several volumes of carbon tetrachlorid. When strained through muslin the pollen passes through with the CCl_4 and can be separated by filtering on filter paper. The pollen is lighter yellow since the CCl_4 probably removed lecithin.

In collecting pine pollen it was found necessary to gather the staminate cones before they had opened, because later the slightest shaking of the branch scattered a cloud of pollen to the four winds. Cutting off the tassels of corn and allowing them to open indoors, over large sheets of paper, undisturbed by currents of air, gave the largest yield of corn pollen.

Preliminary Experiments

These experiments were in two parts: (1) Germination of the pollen grains, and (2) Comparison of the enzyme action of unground, ground, and germinated pollen. The results of these tests showed that the pollen ground with powdered glass was more effective in its enzyme action than either the unground or even the germinated pollen. The experiments were made as follows:

To secure vigorous growth of pollen tubes, Easter lily pollen was germinated (1) in tap water, (2) in 3, 5, and 16 percent sugar solution, (3) on agar, and (4) in Knop's solution and modifications. The stock agar recommended by Crabill and Reed (1915, p. 2) was used. This contains no carbon-containing nutrient and therefore does not favor bacterial and mold growths, which are exceedingly troublesome.

Formula for Stock Agar

Distilled water	1,000 cc.
Magnesium sulphate	0.5 g.
Di-potassium hydrogen phosphate	1.0 g.
Potassium chlorid	0.5 g.
Ferrous sulphate	0.1 g.
Agar	2.0 g.

The pollen tubes grew exceedingly well in the film of moisture formed on the surface of agar in petri dishes. The tubes were thicker, appeared more vigorous, and showed protoplasmic movement better than when grown in water or in dilute sugar solutions. This might be used as a method of showing variation in cell turgescence according to the density of the medium.

Formula for Knop's Solution

K_2SO_4	0.7 g. in 1 liter of water
NaCl	0.23 g.
$CaSO_4$	0.7 g.
$MgSO_4$	0.5 g.
Na_3PO_4:	0.5 g.
NH_4NO_3	(solution 0.0649) 20 cc.

The tubes grew best in solutions from which the K_2SO_4 was omitted, and best of all in one in which the $CaSO_4$ was increased to 1.0 g. The K_2SO_4 seemed to cause disintegration of the tubes after 48 hours, but this evidence is of course very slight and more experiments must be tried to prove anything.

Of the four media used, tap water was selected as the best for Easter lily pollen. The grains germinated and produced long tubes in 24 hours, and the solution contained no foreign matter to be taken into consideration. After the tubes were well grown the pollen mass was filtered and dried in a desiccator. This dried germinated pollen was used both unground and ground with powdered glass.

Comparative quantitative determinations of the enzyme action of the unground, ground, and germinated pollen were made as follows: Having previously noted the marked invertase action of Easter lily pollen on cane sugar, the amount of copper precipitated from Fehling's solution by the reducing sugar formed was taken as an index of enzyme action.

The tests were made in five test tubes as follows: In each tube were placed 300 mg. of cane sugar, 15 cc. of distilled water (except in tube 4, where 10 cc. was used), and 8 drops of toluol. To tubes 1, 2, and 3 were added respectively 300 mg. each of unground, ground, and ground germinated pollen. To tube 4 was added 300 mg. of pollen boiled in 5 cc. of water, making the total quantity the same as in the other tubes. Tube 5 had no pollen added and served as a second control.

These tubes were allowed to stand in a warm room for 24 hours and were shaken occasionally. After this interval, 15 drops of each of the five

suspensions was taken. To each portion 15 cc. of fresh Fehling's solution was added. The tubes were placed in a water bath and boiled an hour. The solutions were then filtered on desiccator-dried, weighed filter paper, and the copper precipitate was washed with hot water until free from the excess of Fehling's solution. The filter papers were then dried first in an oven and then in a desiccator and again weighed. The gain in weight represents the amount of reducing sugar present.

The weights of the papers are shown in table 8.

TABLE 8

	1st Weight	2d Weight	Gain
Unground pollen	824 mg.	857.05 mg.	33.05 mg.
Ground pollen	832 mg.	860.2 mg.	28.2 mg.
Ground germinated pollen	831.5 mg.	857 mg.	25.5 mg.
Boiled pollen	843 mg.	845.35 mg.	2.35 mg.
Sugar solution only	828 mg.	829.1 mg.	1.1 mg.

The gain in the unground pollen, which appears larger, is relatively less because in this test the 300 mg. was all pollen, while the 300 mg. in the other tests was partly powdered glass. From these figures and from several similar tests it seemed evident that in the case of Easter lily pollen invertase, at least, there was no advantage in previously germinating the pollen grains. Repetition of this type of experiment might show a wide range of variation both for kinds of pollen and for their enzymes.

The data obtained in testing for pectinase in Easter lily pollen confirmed the opinion that for this kind of pollen there was no gain in pectinase as a result of germination.

Tests for Amylase

The method used was to test a known quantity of starch paste with active pollen and an equal quantity with boiled pollen for a control. First, 10 cc. of 1 percent starch paste was used with 150 mg. of pollen. Later, 5 drops of 1 percent starch in 10 cc. of water was found to be a better dilution. Toluol was used as an antiseptic. The tubes were allowed to stand in a warm room for 24 hours and were shaken occasionally. Two portions of 15 drops each were then taken from each tube, and to one was added 2 drops of iodine to see if the starch had disappeared, and the other was heated with 15 drops of Fehling's solution to see if sugar had appeared. The results are seen in table 9.

In these tests, as in those already mentioned, the ground pollen was more active than the unground, but the germinated pollen did not appear to be more active than the ungerminated.

From table 10 it is seen that all kinds of pollen tested contained an amylase, but that this amylase was less active in the apple pollen (Siberian crab) and in that of the magnolia (cucumber tree) than in the other kinds.

In later tests with other kinds of pollen, Benedict's solution was used instead of Fehling's solution, as it is a more delicate test.

TABLE 9. *Tests for Amylase in Easter Lily Pollen*

	10 Cc. of 1 Percent Starch Solution + Toluol	5 Drops of 1 Percent Starch Solution in 10 Cc. H_2O + Toluol
Fresh (unground)	Slight digestion	Complete digestion
Fresh (unground)	Marked digestion but not complete	Complete digestion
Germinated	Marked digestion but not complete	Complete digestion
Germinated (ground)	Nearly complete digestion	Complete digestion
Boiled pollen	No digestion	No digestion

TABLE 10. *Tests for Amylase in Different Kinds of Pollen*

Pollen, 150 mg., added to 5 drops of 1 percent starch solution in 10 cc. of water to which toluol was added as an antiseptic.

Tests for starch: 15 drops of starch solution + pollen + 2 drops of iodine.

Kinds of Pollen	Active Pollen	Boiled Pollen
Easter lily	Rapid digestion	No digestion
Lilium rubrum	" "	" "
Red maple	" "	" "
Norway maple	" "	" "
Apple, Siberian crab	Slight digestion	" "
Austrian pine	Rapid digestion	" "
Scotch pine	" "	" "
Cucumber tree	" "	" "
Dandelion	Slow digestion	" "

Tests for sugar: 15 drops of starch solution + pollen, heated with 15 drops of Fehling's solution.

Kinds of Pollen	Active Pollen	Boiled Pollen
Easter lily	Rapid reduction	Some reduction
Lilium rubrum	" "	" "
Red maple	" "	" "
Norway maple	" "	" "
Apple, Siberian crab	Some reduction after ½ hr. heating	No reduction Cf. Table 8
Austrian pine	Rapid reduction	Some reduction
Scotch pine	" "	" "
Cucumber tree	" "	" "
Dandelion	" "	" "

Tests for Reducing Sugars

Since all the controls in the tests of amylase, except the boiled apple pollen, gave some reduction of Fehling's or of Benedict's solution, tests were made to determine the kind of sugar present in pollen. Filtered water extracts of the kinds of pollen listed above were heated with Fehling's solution. All except the apple pollen were found to contain reducing sugars, or some easily oxidized substance. When the apple-pollen extract was

hydrolyzed with HCl and then neutralized with NaOH, it reduced the Fehling's solution, indicating the presence of a sucrose.

Tests for Starch

Solutions were treated first with chloral hydrate to render the grains transparent, and afterwards with iodine.

TABLE 11. *Tests for Starch in Different Kinds of Pollen*

1. Apple	−	10. Pine, Austrian	−
2. Corn	+	11. Pine, white	−
3. Daisy	−	12. Ragweed	−
4. Dandelion	−	13. Rye	+
5. Dock	+	14. Timothy	+
6. Elm	+	15. Magnolia	−
7. Goldenrod	−	16. Maple, Norway	−
8. Lily, Easter	−	17. *Lilium rubrum*	−
9. Lily, tiger	−	18. Maple, red	−

Tests for Zymase

The different kinds of pollen were tested with Pasteur's fluid, in Smith's fermentation tubes, for zymase. Toluol was added to inhibit bacterial action or molds. Apple pollen was the only one which showed any reaction, and since this was after standing 48 hours the result was doubtful. Since, however, apple pollen has been an exception in other instances, this test will be repeated when more pollen is available.

Tests for Invertase

Equal amounts of the different kinds of ground pollen (about 150 mg.) were added to 5 cc. of 3 percent cane sugar solution with 5 cc. of distilled water and 8 drops of toluol. Equal amounts of ground pollen were boiled with 5 cc. of distilled water and added to 5 cc. of the cane sugar with 8 drops of toluol solution for controls. The two sets of tubes were allowed to stand for 24 hours in a warm room. Then to 15 drops of each pollen solution were added 15 drops of Fehling's solution and the tubes were heated ½ hour to 1 hour in a boiling water bath, and the rate and amount of reduction in the different tubes were observed. Although this was not an exact quantitative test, as for the Easter lily pollen, yet the varying amounts of reduction in the different pollen solutions, and the differences between the active solutions and the controls, were strikingly noticeable. When the pollen was acid, producing a green color in Fehling's solution before heating, the tests were repeated, neutralizing the solution first. This was marked in red maple. Since the active pollen in every case caused more reduction than the boiled control, the reduction could not have been due merely to the reducing sugars of the pollen grains since the ruptured boiled grains would have yielded just as much sugar. The difference, therefore, may be

due to the action of invertase. Moreover, apple pollen, which was found to contain sucrose, was extremely active in its invertase reaction. The results are shown in table 12.

TABLE 12. *Tests for Invertase*

Kinds of Pollen	Active Pollen	Control
Easter lily pollen.....	Rapid reduction	Slight reduction
Lilium rubrum.....	Slow "	" "
Red maple.....	Very rapid reduction	" "
Norway maple.....	Rapid reduction	" "
*Apple.....	Instant "	Some after 20 min. heating
Austrian pine.....	Slow but marked	Slight reduction
Scotch pine.....	" " "	" "
Magnolia.....	Very rapid	" "
Dandelion.....	Slow but marked	" "

Tests for Lipase

In the different methods used for testing for lipolytic enzymes the following substrates and testing reagents were used:

1. *Substrates.*

   (1) Ethyl butyrate.

   (2) Olive oil acidified with decinormal acetic acid and a little gum arabic added to make an emulsion.

   (3) Olive oil emulsion recommended by Zeller. 10 cc. of olive oil was dissolved in hot 100 percent alcohol. This was run through a hot separating funnel to which was attached a piece of glass tubing drawn out to a capillary jet. The stream of oil in alcohol was run into 100 cc. of cold distilled water which was stirred continually. The milky emulsion was then heated to drive off the alcohol and afterwards diluted with water.

   (4) Methyl acetate.

2. *Activator.* Approximately N/60 oxalic acid was used, partly because free acid is needed to counteract the slight alkalinity of the ground glass and more especially because free acid accelerates the activity of lipase.

3. *Alkali for titration.* Approximately N/10 sodium hydroxid solution was used to which a trace of barium hydroxid was added. To insure uniformity in readings, a 3-liter bottle was filled, and the solution was drawn off as needed through a connected graduated burette. Both the bottle and the burette had soda-lime bulbs at the inlet to absorb CO_2.

4. *Indicator.* Phenolphthalein was used in all titrations as an indicator.

5. *Antiseptic.* Toluol was added as an antiseptic. Controls of autoclaved pollen extract were run in each case, and the digestions were carried on in small stoppered Erlenmeyer flasks kept in an electric incubator at 36°–38° C. Samples were titrated at different time intervals. Methyl acetate was more strongly hydrolyzed than either ethyl butyrate or the olive oil preparations.

Austrian pine, dock, daisy, goldenrod, ragweed, rye, and timothy pollens were tested with the different substrates for lipase. The tests with ethyl butyrate were unsatisfactory. In the olive oil emulsion and methyl acetate media, Austrian pine, dock, ragweed, and rye pollens gave positive tests for lipase. The action on methyl acetate was especially marked with Austrian pine pollen, in which case the titrations showed nearly double the amount of acid with fresh pollen as compared with the boiled control.

Tests for Proteolytic Enzymes

Substrates for Proteolytic Enzymes

1. *Blood fibrin.* Fresh fibrin from pig's blood was obtained at the slaughter house, and was washed for several hours with a stream of cold water to remove corpuscles. Fairly uniform and compact strands of the fibrin were selected, and portions as nearly equal as possible were placed in test tubes with 10 cc. of distilled water, plugged with cotton, and sterilized for 20 minutes in an autoclave. Other portions were stained with 1 percent Congo red and the color was fixed by immersion in boiling water. The red color is liberated when the fibrin is digested. The colored fibrin was also sterilized.

TABLE 13. *Fermi's Gelatin Test*

5 cc. Fermi's gelatin, 5 cc. H_2O, 100 mg. pollen, 37° C. Degrees of liquefaction or failure to solidify, after standing in ice water 10 minutes, indicated by signs.

Kind of Pollen	Unheated Pollen		Autoclaved Pollen	
	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.
1. Apple	+	++	−	−
2. Corn	+	+	−	−
3. Daisy	+	+	−	−
4. Dandelion	+	+	−	−
5. Dock	++	+++	−	−
6. Elm	+	++	−	−
7. Goldenrod	+	++	−	−
8. Lily, Easter	++	+++	−	−
9. Lily, tiger	++	++	−	−
10. Pine, Austrian	+	+++	−	−
11. Pine, white	+	++	−	−
12. Ragweed	+++	++++	−	−
13. Rye	++	++	−	−
14. Timothy	+	+	−	−
15. Magnolia	++	+++	−	−
16. Maple, Norway	+	+	−	−

2. *Fermi's gelatin.* The proportions used were those given by Dernby. 700 grams of gelatin were dissolved in 1,250 cc. of hot water over a water bath, strained through cheese cloth, and 2 grams of finely pulverized thymol were added. The solution was diluted to 2 liters and sterilized. Dernby diluted further before using, but this was not found necessary with the pollen extracts. When the gelatin was used it was melted over a bath and

to 5-cc. portions were added 5 cc. of distilled water and 100 mg. of pollen. For the control the pollen and water were first autoclaved. The tubes were incubated at 37° C. for 24 hours or longer (see table 13). The tubes were taken from the incubator and placed simultaneously in ice water, and the failure to solidify, or degree of congealing, during 10 minutes was noted.

Many investigators have used gelatin for detecting enzymes of the pepsin type, but the experiments of Malfitano, Mavrofannis, and Jordan, recently confirmed by Berman and Rettger, seem to indicate that liquefaction of gelatin by an organism is not proof of proteolytic activity. However, since pollen extracts, like pineapple juice, possess this power of liquefaction to a marked degree it has been considered worth while to record the observations.

Tests for Trypsin

TABLE 14

Congo red, blood fibrin, 2 cc. N/10 Na_2CO_3, 10 cc. pollen extract (50 mg. in 100 cc. distilled water, unheated and autoclaved), 1 mg. thymol in each tube.

Kind of Pollen	Test 24 Hrs.	Appearance of Solutions	
		Unheated	Autoclaved
1. Apple	−	No change	No change
2. Corn	++	Fibers disintegrated. Liquid very pink	Fibers unaltered Liquid pale yellow
3. Daisy	−	No change	No change
4. Dandelion	−	" "	" "
5. Dock	?	Fibers less firm. Liquid pinkish	No change
6. Elm	?	Liquid faint pink	" "
7. Goldenrod	++	Fibers disappeared. Liquid red-brown	Fibers unaltered Liquid yellow-brown
8. Lily, Easter	−	No change	No change
9. Lily, tiger	−	" "	" "
10. Pine, Austrian	+	Fibers partly disintegrated, liquid pink	No change
11. Pine, white	+	Fibers partly disintegrated, liquid pink	No change
12. Ragweed	+	Fibers partly disintegrated, liquid pink	No change
13. Rye	+	Fibers partly disintegrated, liquid pink	No change
14. Timothy	+	Fibers partly disintegrated, liquid pink	No change
15. Magnolia	++	Fibers disintegrated, liquid pink	No change
16. Maple, Norway	−	No change	" "

From tables 14–16 it may be seen that corn, goldenrod, Austrian pine, white pine, ragweed, rye, and timothy all gave positive results. Dock gave strongly positive results in the less alkaline medium, while magnolia and goldenrod were strongly positive in the more alkaline medium. Apple was negative with Na_2CO_3 added, but was positive without the addition of

TABLE 15

Same as shown in table 14, except that no Na_2CO_3 was added. Solutions slightly alkaline from the powdered glass. Thymol added to each tube.

Kinds of Pollen	Test 24 Hrs.	Appearance of Solutions	
		Unheated	Autoclaved
1. Apple	+	Fibers slightly disintegrated. Liquid pink	No change
2. Corn	++	Fibers completely disintegrated. Liquid pink	" "
3. Daisy	?	Fibrin darker, liquid milky	" "
4. Dandelion	−	No change	" "
5. Dock	++	Fibers completely disintegrated. Liquid pink	" "
6. Elm	?	Slight turbidity	" "
7. Goldenrod	+	Fibrin shrunken. Liquid dark brown	" "
8. Lily, Easter	−	" " " " "	" "
9. Lily, tiger	−	" " " " "	" "
10. Pine, Austrian	+	Disintegration, turbid, pink	" "
11. Pine, white	+	" " "	" "
12. Ragweed	+	" " "	" "
13. Rye	+	" " "	" "
14. Timothy	++	Complete disintegration. Liquid red	" "
15. Magnolia	−	No change	" "
16. Maple, Norway	?	Liquid turbid pinkish	" "

Tests for Pepsin

TABLE 16

Same as shown in table 14, except that 2 cc. of 0.2 percent HCl was added instead of Na_2CO_3.

Kind of Pollen	Test 24 Hrs.	Appearance of Solutions	
		Unheated	Autoclaved
1. Apple	−	No change	No change
2. Corn	+	Disintegration. Liquid pink	" "
3. Daisy	−	No change	" "
4. Dandelion	−	" "	" "
5. Dock	−	" "	" "
6. Elm	−	" "	" "
7. Goldenrod	−	" "	" "
8. Lily, Easter	−	" "	" "
9. Lily, tiger	−	" "	" "
10. Pine, Austrian	−	" "	" "
11. Pine, white	−	" "	" "
12. Ragweed	−	" "	" "
13. Rye	+	Disintegration. Liquid pink	" "
14. Timothy	+	" " "	" "
15. Magnolia	−	No change	" "
16. Maple, Norway	−	" "	" "

alkali. It may also be noted that in the digestion of fibrin in the presence of 0.2 percent HCl only the Gramineae showed activity. When Na_2Co_3 was left out, apple, daisy (?), and dock were added to the list. These tests were repeated several times and gave consistent results. Toluol was substituted for thymol without any noticeable difference. In no case was there the slightest odor of putrefaction. The antiseptics were easily detected by their odor.

Tests for Erepsin

Solutions of Witte's peptone were used in the following proportions:

1. 10 cc. of 1 percent Witte's peptone, 2 cc. of N/10 sodium carbonate, 5 cc. of pollen extract 50 mg. in 10 cc. (unheated and autoclaved), 100 mg. of thymol.
2. 10 cc. of 1/10 per cent Witte's peptone, 1 cc. of N/10 sodium carbonate, 10 cc. of pollen extract (unheated and autoclaved), 100 mg. of thymol.
3. The above described solutions were used without adding sodium carbonate.

For testing, Gies's biuret reagent was used. This reagent consists of 10 percent KOH solution, to which 25 cc. of 3 percent $CuSO_4$ solution per liter is added. A large flask was filled with the reagent and connected with a graduated burette so that for each test the same strength of reagent should be used. In making the tests, 1 cc. of the solution to be tested was put with 20 cc. of biuret reagent in 25 cc. Nessler comparator tubes of uniform diameter and thickness. The color differences were read by looking down through the liquid at a white background. Solution 2 proved the best dilution. More than 1 cc. of the pollen-peptone solution did not give satisfactory results because of color interference and turbidity. In each test three tubes were compared: (1) 1 cc. peptone solution, or peptone plus Na_2CO_3, and thymol. (2) 1 cc. peptone, or peptone plus Na_2CO_3 plus unheated pollen, and thymol. (3) 1 cc. peptone, or peptone plus Na_2CO_3 plus autoclaved pollen.

The sixteen varieties of pollen previously listed were tested, but only apple and magnolia pollen gave positive results. Here the reaction of the unheated pollen with the biuret reagent gave a very faint pinkish tint as compared with the rose-violet tint of the controls.

Tests for Catalase

The decomposition of hydrogen peroxid in a fermentation tube was used as an indication of a catalase. All the kinds of pollen tested showed a marked reaction. Easter lily, magnolia, and apple pollen were exceedingly active. Maple pollen was the slowest but the action was evident. The boiled pollen extracts did not give the reaction.

Tests for Reductase or a Reducing Substance

The reduction of potassium permanganate solution by the different kinds of pollen was tested. All showed some reducing action; apple, Austrian pine, and magnolia were especially active. Apple pollen changed $KMnO_4$

at once to a pale amber. The boiled controls did not reduce the permanganate. This is not necessarily indicative of enzyme action. The reduction may be brought about by substances produced by the decomposition of the pollen grains.

Tests for Nuclease

Tests for phosphoric acid, which might indicate the splitting of nucleic acid, were made with ammonium molybdate on ground, unground, germinated, and boiled Easter lily pollen. All showed a strong phosphoric acid test so that no conclusions could be drawn.

Tests for Tyrosinase

A solution of tyrosin gave a negative reaction for all pollens of the first series tested.

Tests for Laccase

An alcoholic solution of gum guiacum, which was rapidly colored blue by freshly cut potato or orange peel, gave negative results with ten varieties of pollen.

Tests for Cytase

1. The method given by Crabill and Reed was used. Filter paper is dipped in manganese sulphate solution and then in potassium permanganate solution. The resulting manganic oxid colors the paper dark brown. Acids formed by the cellulose destruction combine with the manganic oxid to form light-colored salts which show by contrast on the brown background. Both ground and unground pollen placed on moistened sterilized strips of such paper caused no color change, although a subsequent growth of mold did so.

2. Cellulose was prepared from filter paper in the following manner. Schweitzer's reagent (ammoniacal cupric oxid) was used as a solvent. This was made by adding to a strong copper sulphate solution, first, ammonium chloride, and then an excess of sodium hydroxid. The blue-green precipitate thus formed was allowed to settle, the liquid decanted off, and the precipitate washed repeatedly with water on a Buchner funnel and filtered by suction. The precipitate was then dissolved in 0.92 percent ammonia. The resulting deep blue liquid readily dissolves strips of filter paper. When sufficient paper had been dissolved to make a thick, syrupy liquid, it was poured into dilute hydrochloric acid (1 : 5) and the cellulose was precipitated in small flecks. The precipitated cellulose was washed repeatedly with water on a Buchner funnel and filtered by suction, until the filtrate showed no trace of HCl when tested with $AgNO_3$. The pure white mass of cellulose was then boiled with distilled water to make a fine suspension and to sterilize it. The suspension was tested with both iodine and Benedict's solution to be sure that it was both starch- and sugar-free, as was the case. The tests were made as follows: 10 cc. of cellulose suspension, 100 mg. of ground

pollen, 30 cc. of distilled water, and 2 cc. of toluol were placed in small stoppered flasks. For the control the pollen and water were heated in the autoclave. The suspensions were incubated at 37° C., and shaken frequently. The solutions were tested for sugar at 24-, 48-, and 96-hour intervals, and then allowed to stand for several weeks at room temperature to see if there would be complete destruction of the cellulose. In no case has this occurred, although several preparations have been kept for three months (see table 17). In testing for sugar the flasks were well shaken. Then the cellulose was allowed to settle out and 2 cc. of the clear liquid was transferred to a test tube and 10 cc. of Benedict's solution was added. All the tubes, both the unboiled and the boiled pollen controls, were then heated simultaneously in a boiling water bath until reduction was complete. If there was a striking difference in the amount of precipitate in the unboiled and in the control the quantitative test was made, but if no difference could be detected the precipitates were weighed as described on a previous page. Pollens were selected which did not all contain starch, so that the resulting gain in sugar did not come from this source. Yet the diminution in the amount of cellulose in the flasks was so slight that it is difficult to interpret results.

TABLE 17

Kinds of Pollen	Unheated Pollen	Autoclaved Pollen
1. Apple	+	−
2. Corn	−	−
3. Daisy	−	−
4. Dandelion	−	−
5. Dock	+	−
6. Elm	−	−
7. Goldenrod	−	−
8. Lily, Easter	−	−
9. Lily, tiger	−	−
10. Pine, Austrian	+	−
11. Pine, white	+	−
12. Ragweed	−	−
13. Rye	+	−
14. Timothy	+	−
15. Magnolia	−	−
16. Maple, Norway	−	−

Tests for Pectinase

As has already been noted, special importance was attached to the possible occurrence of a pectinase as indicative that the pollen tube digests the inner lamella of pectin in the cell walls of the pistil. Three methods of testing for a pectinase were tried.

1. Pistils of Easter lily were placed in a tube with distilled water and freshly ground pollen, and a second set were similarly treated with boiled pollen. After 24 hours the pistils were examined for alteration of texture.

It was found that the pistils with the active pollen had noticeably softened and the styles could easily be teased apart with dissecting needles, while the pistils with boiled pollen were still firm. The longitudinal and cross sections of the pistil treated similarly showed the same results. Incidentally, the chemotropism of the pollen grain for the stigma was conspicuous in the active pollen tubes.

2. Equal amounts of desiccator-dried ground Easter lily pistil were treated with pollen, and tested quantitatively for sugar as in the invertase test already described.

	Dried Ground Pistil	Boiled Pollen	Water	Toluol
(*a*)	150 mg.....	100 mg. in 5 cc. water	10 cc.	4 cc.
	"	100 mg. ungerminated	15 cc.	"
	"	100 mg. germinated	"	"
	"	100 mg. ground	"	"

(*b*) Solution 15 drops, alkaline to neutral.
Fehling's solution 15 drops.
Boil in water bath ½ hour.
Filter on weighed, desiccator-dried paper.
Dry in oven and desiccator.
Weigh again.

	1st Weight (mg.)	2d Weight (mg.)	Gain (mg.)
(*c*) Boiled pollen.....	711	716.3	5.3
Ungerminated.....	711.2	720.15	8.95
Germinated.....	720.28	729.00	8.72
Ground.....	827.00	839.00	12.00

3. The action of pollen on pectin was tested. The pectin used for the tests was prepared from the white inner skin of grape fruit, as follows:

Remove the white and boil in water.
Put through a meat chopper.
Leave in cold water 24 hours.
Boil from ½ to 1 hour.
Strain through cheesecloth.
Filter.
Concentrate by heating over a water bath.
Add 95 percent alcohol to precipitate.
Filter.
Wash with absolute alcohol.
Dry in a desiccator.

For the tests the following amounts were used:

(*a*) 3 cc. of pectin solution (300 mg. in 20 cc. of water), 300 mg. of pollen, 15 cc. of water, 8 drops of toluol.

(*b*) 15 drops of the pollen-pectin solution, 15 drops of Fehling's solution, or Benedict's solution. Treat as stated above (2 *b*).

	1st Weight (mg.)	2d Weight (mg.)	Gain (mg.)
(*c*) Austrian pine (active)	767.5	789	21.5
" " (boiled)	779	785	6
Norway maple (active)	778	797	19
" " (boiled)	782	794	12
Magnolia (active)	814	835	21
" (boiled)	809	815	6
Apple (active)	795	805	10
" (boiled)	799	807	8

(*d*) In this test 150 mg. of pectin and 150 mg. of ground pollen and glass were used, and the entire amount precipitated with Fehling's solution.

	1st Weight (mg.)	2d Weight (mg.)	Gain (mg.)	Actual Gain (mg.)	
Apple pollen (active)	765	963	198(−150)	48	28
" " (boiled)	754	924	170(−150)	20	
Red maple (active)	767	969	202(−150)	72	23
" " (boiled)	748	947	199(−150)	49	

(*e*) Later similar tests were made with seven other kinds of pollen, daisy, dock, goldenrod, white pine, ragweed, rye, and timothy. All gave positive results for the pectinase test.

Subtracting the constant 150 mg. of pollen and glass, the actual gain of sugar from pectin for the apple pollen, as compared with the control, is 28 mg. and for the red maple pollen 23 mg. This is larger than in the previous table, but larger quantities were used. Not only do the quantitative determinations show that pectin is converted into sugar by active pollen, but also in comparing the tests and their controls in the test tube it was very noticeable that the reduction of Fehling's solution was greater with unboiled pollen.

4. Another test which confirmed the presence of a pectinase was made for me by Mr. F. B. H. Brown, Yale University. Mr. Brown in his work on tropical woods, by a special method of technique, has succeeded in making sections one eighth as thick as are usually cut. When sections of dragon-tree wood (*Dracaena aurea*), *Tecoma obtusa*, and a species of roselle were floated on water with Easter lily pollen and allowed to remain from 24 to 48 hours, on examination with the microscope it could be plainly seen that in many places the middle lamellae of the cells had been completely digested. Permanent slides were prepared.

Tests for Bacteria and Molds on Pollen

1. One method was as follows: Extracts of nine different pollens were made, 50 mg. in 10 cc. of water. This extract was then diluted by the usual milk-testing method in sterile bottles to dilutions of 1 : 100 and 1 : 10,000. 1 cc. of this dilution was then placed in sterile petri dishes and agar plates were poured. The plates were then incubated at 37° for 24 hours, in an inverted position to prevent moisture from washing off cultures,

and the colonies were counted. Results from two of several tests are shown, giving the averages (see table 18).

2. 100 mg. of Easter lily pollen was ground well with sterile sand, then well shaken with 100 cc. of 0.85 percent NaCl and 100 mg. of thymol. 1 cc. of this suspension was diluted with 100 cc. sterilized water. Plates of agar were poured with 1 cc. of this dilution and incubated 24 hours at 30°. The average number of colonies on 4 plates was 3. The experiment was repeated, omitting thymol, and the average number on 4 plates was 12. Similar tests with corn, ragweed, and rye gave corresponding results. Thymol does exert an inhibiting action but does not prevent growth of bacteria introduced with the pollen.

3. One gram each of corn, ragweed, rye, and Easter lily pollen was ground with sterile sand and extracted 24 hours in the ice chest with 100 cc. of 0.85 percent NaCl. This solution was filtered through a small, thoroughly sterilized Berkefeld filter into a sterile side-neck flask. Care was taken to plug the side-neck with cotton before sterilizing so that no contamination could enter while filtering by suction. 1-cc. portions of each filtrate were removed with a sterile pipette and agar plates were poured, 4 of each kind of pollen. These were incubated at 37° for 48 hours. No colonies appeared. All the plates were sterile. Six days later one plate had a colony of mold, but this could easily have been later contamination. The sterile filtered extracts were then used for testing for diastase, liquefaction of gelatin, and digestion of fibrin. The diastatic action seemed as rapid as before filtration, but the gelatin liquefaction and fibrin digestion were decreased. The gelatin was completely liquefied after 48 hours, and the rye and ragweed extracts caused only slight digestion of the fibrin. Repetition with other samples confirmed the belief that the filter absorbed the enzyme to a considerable extent. If one could use separate filters for each kind of pollen and work with large quantities, this difficulty might be overcome by allowing the filter to become saturated with the extract.

TABLE 18. *Bacteria and Mold Colony Counts. Averages of Four Plates each. Dilutions 1 : 100 and 1 : 10,000. 37°, 24 Hours.*

Kind of Pollen	Unheated		Autoclaved	
	1 : 100	1 : 10,000	1 : 100	1 : 10,000
Corn	8	0	0	0
Daisy	17	0	0	0
Dock	72	2	14	1
Goldenrod	20	0	0	0
Lily, Easter	?	0	0	0
Pine, Austrian	11	0	0	0
Ragweed	43	1	0	0
Rye	31	2	0	0
Timothy	27	14	0	0

4. Washing the pollen before use was tried with corn, ragweed, and Easter lily. 1 gram of unground pollen was shaken vigorously with 300 cc. of distilled water in a liter cylinder, and then filtered into a sterile flask through a sterilized Buchner funnel and filter paper, and washed four times with sterile distilled water. Plates were poured from the last washing, dilution 1 : 100. Some plates were sterile and others from the same filtrate had from 1 to 19 colonies. The washed pollen when diluted and tested showed a similar lack of constancy, so that there seems to be no gain in washing, and this method may cause loss of enzymes.

Repetition with dock pollen failed to show that it always had a higher count. There was probably some initial contamination in preparing the extract.

SUMMARY

Although it has been assumed that pollen tubes digest their way through the style, there is little experimental evidence as to the exact nature of this enzyme action. Histological examination shows that in most instances pollen tubes make their way between the walls of adjacent cells rather than penetrating them. We should expect therefore to find most frequently not a cytase- or cellulose-digesting enzyme, but rather a pectinase capable of digesting the pectin of the inner lamella. This has been proved in the writer's experiments to be the case.

Eighteen species of pollen have been used; these have been tested for thirteen kinds of enzymes. So far amylase, invertase, catalase, reductase, and pectinase have been found in all. Pepsin, trypsin, erepsin, and lipase have been demonstrated in some and not in others. Cytase was doubtfully identified in six of the eighteen. Tyrosinase and laccase have not been found in any, and zymase was found only in Siberian crab apple pollen.

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